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CERTIFICATE

I hereby certify that annexed is a true copy of the PCT International Application number PCT/NZ99/00160 filed on 24 September 1999 by GLYCOX CORPORATION LIMITED.

Dated 3 July 2003.

Neville Harris
Commissioner of Patents

COMMISSIONER OF PATENTS, TRADE MARKS & DESIGNS
NEW ZEALAND

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT/NZ99/ 00160

International Filing Date 24 SEP 1999 (24-09-1999)

NEW ZEALAND PATENT OFFICE
P.C.T INTERNATIONAL APPLICATION
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) P409692 DJJ

Box No. I TITLE OF INVENTION

FRUCTOSAMINE OXIDASE ASSAY : METHODS AND MATERIALS

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

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☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

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Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ AP **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

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- ☒ BB Barbados
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- ☒ KP Democratic People's Republic of Korea
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- ☒ LK Sri Lanka
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PCT/NZ99/ 00160

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) (25.09.98) 25 September 1998	332085	NEW ZEALAND		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): 1

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

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Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	:	3
description (excluding sequence listing part)	:	12
claims	:	3
abstract	:	1
drawings	:	3
sequence listing part of description	:	
Total number of sheets	:	22

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract: 1

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

DAVID JOHN JONES
Agent for the Applicant

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1. Date of actual receipt of the purported international application: 24 SEP 1999 (24/09/99)
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
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FRUCTOSAMINE OXIDASE ASSAY: METHODS AND MATERIALS

THE CURRENT INVENTION

The present invention relates to methods and materials for the assay of fructosamine oxidase enzyme in patients and particularly but not solely those predisposed to or with diabetes mellitus.

Diabetes mellitus is a common disease characterised by serious long-term vascular complications. Diabetic individuals have a 25-fold increase in the risk of blindness, a 20-fold increase in te risk of renal failure, a 20-fold increase in the risk of amputation as a result of gangrene, and a 2- to 6-fold increased risk of coronary artery disease and ischemic brain damage. See, Klein R, Klein B, Davis M, DeMets D. *Diabetes Care* 8:311-5 (1985). Almost half those diagnosed as diabetic before the age of 31 years, die before they reach 50 years largely as a result of cardiovascular or renal complications, often with many years of crippling and debilitating disease beforehand. See, Deckert T, Poulsen J, Larsen M. *Diabetologia* 14:363-70 (1978).

Elevated blood glucose levels are now regarded as ***causative*** of diabetic complications based on results of the Diabetes Complications and Control Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS). See, The Diabetes Control and Complications Trial Research Group. *N Eng J Med.* 379:977-85 (1993) and *Lancet* 352:837-53 (1998). The DCCT and the UKPDS have both demonstrated that the development of complications of diabetes are related with degree of hyperglycaemia and that long-term outcome may be ameliorated by rigorous treatment. After controlling for current HbA_{1c} levels, the development of micro vascular complications in DCCT patients was strongly correlated with the degree of nonenzymatic glycation of structural proteins such of skin collagen, but not with advanced glycation end product (AGE) markers such as pentosidine, carboxymethylysine, and tissue fluorescence (V Monnier - personal communication). These findings imply that the nonenzymatic glycation of tissue proteins has greater pathophysiological importance than AGE formation.

Many of the features of diabetic vascular disease may also be attributed to oxidative stress, defined as an increase in the steady-state level of reactive oxygen or oxygen radicals in a biological system See, Baynes JW. *Diabetes* 40;405-12 (1991). For

example, superoxide anions increase intracellular calcium which modulates the activity of nitric oxide synthase in the endothelium. Nitric oxide is a potent vasodilator and it has been implicated in the vascular dysfunction of early diabetes See, Ido Y, Kilo C, Williamson JR. *Nephrol Dial Transplant* 11 Suppl 5:72-5 (1996). Reactive oxygen species precipitate a drastic dose-dependent decrease in *de novo* synthesis of heparan sulphate proteoglycans leading to a reduction in anionic sites on the basement membrane and to an increased permeability to positively charged proteins such as albumin See, Kashira N, Watanabe Y, Makin H, Wallner EI, & Kanwar YS. *Proc Natl Acad Sci USA* 89:6309-13 (1992). Such leaky capillaries manifest clinically as background retinopathy and microalbuminuria. Microalbuminuria, in turn, is a recognised risk factor both for diabetic nephropathy in IDDM and for coronary artery disease and sudden death in elderly NIDDM See, Mogensen CE, Christensen CK. *N Eng J Med* 311;89-93 (1984) & Mogensen CE, Damsgaard EM, Froland A, et al *Clin Nephrol* 38 (suppl 1);s28-39 (1992).

Once natural anti-oxidant defences are exceeded, there is the potential for hydroxyl radical generation from superoxide via a copper catalysed Haber-Weiss reaction See, Halliwell B & Gutteridge JMC "Free radicals in Biology and Medicine" Clarendon Press, Oxford (pp. 136-76 1989). Hydroxyl radicals are extremely reactive species that cause serious site-specific damage.

Oxygen radicals have also been implicated in the oxidative modification of low density lipoprotein (LDL) See, Witztum JL. *Br Heart J* 69; S12-S18 (1993). Oxidised LDL is a specific risk factor for atherosclerosis, binding with a scavenger receptor on tissue macrophages leading to the formation of foam cells and to cholesteryl ester accumulation in the intimal fatty streak, a feature of atheromatous plaque formation.

To date, the source of the oxidative stress in diabetes has not been identified. I have isolated a novel extracellular enzyme which catalyses the elimination of fructosamines from glycated protein. The existence of this enzyme has not previously been recognised in the world literature. The reaction is important because fructosamine is the precursor of all the Maillard products. Based on its high specificity for glycated protein substrates and its use of oxygen as acceptor, the enzyme may be classified as **fructosamine oxidase 1.5.3** See, Enzyme nomenclature, Recommendations of the

Nomenclature Committee of the International Union of Biochemistry, Academic Press, London pp. 19-22, (1979). Fructosamine oxidase is a metalloenzyme with copper and quinone cofactors. Reaction products are free unglycated protein, α-dicarbonyl sugar, and superoxide (Figure 1).

SUMMARY OF THE INVENTION

The existence of the fructosamine oxidase enzyme has not previously been recognised in the world literature. This is a novel enzyme. The present invention relates to methods of monitoring fructosamine oxidase inhibition and/or antagonism of patients, methods for testing or identifying fructosamine oxidase inhibitors, methods of screening patients to determine patients at risk to vascular (particularly microvascular) damage and methods of identifying those individuals who will benefit by treatment with fructosamine oxidase inhibitors and/or antagonists, methods of determining fructosamine oxidase levels in a mammal, methods of determining blood plasma fructosamine oxidase levels in a diabetic individual or a suspected individual, methods of assaying blood serum or blood plasma *in vitro* for fructosamine oxidase and to related methods and procedures.

In one aspect the invention consists in a method of determining fructosamine oxidase activity in blood plasma of mammalian patients or a mammalian patient to determine patients or a patient at risk to vascular damage, which method comprises determining the levels of fructosamine oxidase and/or the superoxide reaction product of fructosamine oxidase and/or any other oxygen free radical product of fructosamine oxidase in the population of patients and making the determination dependant upon such levels.

Preferably the patients are humans suffering from or predisposed to diabetes.

Preferably said fructosamine oxidase activity is measured in blood taken from each patient.

Preferably the measurement conducted *in vitro* is of the superoxide reaction product or any other oxygen free radical product of fructosamine oxidase.

Preferably at risk patients are or an at risk patient is then treated inter alia to inhibit and/or to antagonise fructosamine oxidase.

Accordingly, in another aspect the present invention consists in a method of screening mammalian patients (preferably humans suffering from or predisposed to diabetes) to determine patients at risk to vascular (particularly microvascular) damage, which method comprises determining the levels of fructosamine oxidase and/or the superoxide reaction product (or any other oxygen free radical product) of fructosamine oxidase in the population of patients and making the determination dependant upon such levels.

Preferably said screening is of blood taken from each patient.

Preferably the measurement conducted *in vitro* is of the superoxide reaction product (or any other oxygen free radical product) of fructosamine oxidase.

Preferably at risk patients are then treated inter alia to inhibit and/or to antagonise the fructosamine oxidase.

Preferably the procedure is substantially as hereinafter described.

In still a further aspect, the present invention consists in a method of identifying those individuals who will benefit by treatment with fructosamine oxidase inhibitors and/or antagonists, which method comprises testing an individual or a group of individuals for fructosamine oxidase in their blood directly or by reference to the superoxide reaction product (or any other oxygen free radical product) of fructosamine oxidase.

Preferably at risk patients are then treated inter alia to inhibit and/or to antagonise the fructosamine oxidase.

Preferably the procedure is substantially as hereinafter described.

In still a further aspect, the present invention consists in a method of monitoring fructosamine oxidase inhibition and/or antagonism of a patient which comprises or includes testing (directly or indirectly) the fructosamine oxidase level of such patient.

Preferably such testing is by reference to the superoxide reaction product (or any other oxygen free radical product of fructosamine oxidase) in the blood of the patient.

Preferably each of the methods involves a determination of a particular level attributed to fructosamine oxidase and/or the reaction products referred to in comparison to such level or levels of a patient or patients (as the case may be) who is or are not at risk to such vascular damage, or will not benefit by treatment with

fructosamine oxidase inhibitors and/or antagonists or have no need for fructosamine oxidase inhibition and/or antagonism.

In another aspect the invention consists in a method of testing and/or identifying fructosamine oxidase inhibitors or a fructosamine oxidase inhibitor which comprises measuring the effect a candidate substance has or candidate substances have on one or more of the quinone co-factor, or the copper co-factor of fructosamine oxidase.

In yet another aspect the invention consists in a method of identifying a candidate substance for trial for the amelioration of diabetes induced vascular damage in a mammal which comprises testing such a substance for fructosamine oxidase inhibition and/or antagonism and choosing to trial the substance where

(i) it has a specifity for such an enzyme or its co-factors

and

(ii) it has an effectiveness for such inhibition and/or antagonism at dosage levels not known to be toxic or contraindicated in such a mammal.

In still a further aspect the present invention consists in the measurement *in vitro* of the superoxide reaction product (and/or any other oxygen free radical product) of fructosamine oxidase in the blood of a mammal by exploiting its reductant properties or its oxidant properties or by enzymatic means.

In one preferred form said measurement procedure involves (preferably at a pH 7 to 8 (most preferably at pH greater than 7.5)) the disabling of the superoxide scavenging mechanism (such as superoxide dismutase) (SOD) [e.g. using potassium cyanide or (more preferably) by pretreatment with antihuman CuZn SOD antisera] and then exposure [e.g. by addition] to a suitable fructosamine oxidase substrate [e.g. glycated bovine serum albumin modified to eliminate copper chelating activity which might disable the fructosamine oxidase].

Preferably the measurement following from the preferred procedure described involves a consideration [e.g. measurement] of an absorbance change, chemiluminescent change, or some other characterising change in an indicator of the modified sample.

In still a further aspect the present invention consists in a method of determining the fructosamine oxidase levels in a mammal (human or non-human) which at least

includes procedures as previously set forth.

In still a further aspect the present invention consists in a method of determining blood plasma fructosamine oxidase levels in a diabetic individual or a suspected diabetic individual which comprises at least steps of a method as previously set forth.

In still a further aspect the present invention consists in a method of assaying blood serum or blood plasma *in vitro (directly and/or indirectly)* for fructosamine oxidase which involves at least one or more of the steps or procedures hereinbefore described and/or hereinafter described.

In still a further aspect the present invention consists in a blood serum or blood plasma sample of a patient in which the superoxide scavenging mechanisms therein have been disabled and the pH is in the range from 7 to 8.

Preferably said sample also includes or has been modified by exposure to a suitable fructosamine oxidase substrate.

Preferably said fructosamine oxidase substrate is glycated bovine serum albumin modified to eliminate copper chelating activity which might disable fructosamine oxidase.

In still a further aspect the present invention consists in the use of a sample in accordance with the present invention for the purpose of any of the methods previously set forth.

The attention of the reader is drawn to my simultaneously filed PCT Application (claiming New Zealand priorities from NZ 332084, NZ 332079 and NZ 334471) in which there are disclosed a variety of procedures, methods, pharmaceutical compositions, dosage units etc. involving the use of fructosamine oxidase inhibition and/or antagonism in order to reduce vascular (preferably microvascular) damage to patients (particularly although not solely diabetic or suspected diabetic patients).

Preferably any such inhibitor or antagonist is selected from the groups

(i) copper chelating agents (eg: triethylenetetramine dihydrochloride, penicillamine, sar, diamsar, ethylenediamine tetraacetic acid, *o*-phenanthroline and histidine)

(ii) substrate analogues (eg: *N*-acetylcysteine, captropril and enalapril).

(iii) hydrazine compounds (eg: diaminoguanidine, hydralazine and

carbidopa).

As used herein including the appended claims the term "and/or" means "and" or "or".

The full content of the simultaneously filed PCT International patent specification is hereby included by way of cross reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a detailed reaction mechanism for the formation of fructosamine and Maillard products from glucose and protein. Fructosamine oxidase degrades fructosamine by a two-step reaction with initial release of an α-dicarbonyl sugar and subsequent oxidation of the enzyme/protein complex to release free unglycated protein. The reduced copper cofactor is oxidised *in vivo* by molecular oxygen and the oxidation product is superoxide.

Figure 2 shows the relationship between *fructosamine oxidase* measurements and plasma fructosamine. Linear regression equation ($y = 0.0349x - 5.9589$; $r^2 = 0.7455$).

Figure 3 shows the effect of *fructosamine oxidase* inhibitors on enzyme activity in human blood plasma. The three inhibitors are selected, merely by way of example, from the three classes of compounds which inhibit the enzyme (i.e. captopril is a substrate analogue, carbidopa is a hydrazine compound, and potassium cyanide is a copper chelator).

Detailed Description of the Invention

(i) Assay principle

Fructosamine oxidase catalyses the degradation of fructosamine(s) with concurrent reduction of molecular oxygen yielding a superoxide reaction product (FIGURE 1). Superoxide is unstable in aqueous solution with spontaneous dismutation to hydrogen peroxide and oxygen. The dismutation reaction is strongly pH dependent with maximal reactivity in acidic solutions and reducing reactivity in alkaline solution. Therefore, enzyme activity is best determined at pH values 7.0-8.0 and preferably about pH 7.5 where superoxide is more stable using one of the assay compounds listed in Table 1.

TABLE 1

Assay compound	Assay pH	Type of reaction	Reference
Ferricytochrome c	7.8	Reduction	McCord J & Fridovich I . *J Biol Chem* 244;6087-93 (1969)
Nitroblue tetrazolium	7.8	Reduction	Halliwell B *FEBS Lett* 72;8 (1976)
Dichlorphenol indophenol	7.0	Reduction	Greenstock CL & Ruddock GW. *Int J Radiat Phys Chem* 8;367 (1976)
Epinephrine	7.8	Oxidation	Misra HP & Fridovich I *J Biol Chem* 247;3170-5 (1972)
Hydroxylamine	7.8	Oxidation	Elstner EF, Heupel A. *Anal Biochem* 70;616-20 (1976)
Peroxidase	7.8	Enzymatic	Misra HP, Fridovich I *Anal Biochem* 79;553-60 (1977)
NADH...LDH	7.0	Enzymatic	Chan PC & Bielski BHJ. *J Biol Chem* 249;1317-9 (1974)
NADH...GDH	7.2	Enzymatic	Chan PC & Bielski BHJ. *J Biol Chem* 255;874-6 (1980)

(ii) Interference

Because superoxide is potentially a noxious substance, superoxide degrading enzyme, superoxide dismutase (SOD), is elaborated in plasma as a physiological response to increasing superoxide concentrations. Compared with healthy non-diabetic individuals, SOD levels are significantly elevated in the plasma of patients with diabetes mellitus and particularly amongst those patients with microvascular disease such as diabetic nephropathy and diabetic retinopathy. See, Mizobuchi N, Nakata H, Horimi T, Takahashi I. *Rinsho Byori* 41;673-8 (1993). The major SOD isoenzyme in extracellular fluids like plasma is extracellular SOD which is a tetrameric glycoprotein that contains four copper atoms and four zinc atoms. See, Karlsson K & Marklund SL *Biochem J* 242;55-9 (1987). Unless it is disabled, such SOD activity will cause significant interference in any blood plasma assay based on the detection systems listed in Table 1.

Almost all of the SOD activity of human plasma is sensitive to inhibition with millimolar concentrations of potassium cyanide, sodium azide or sodium fluoride. Alternatively, SOD activity of plasma may be eliminated by pre-treatment of the plasma sample with anti-human CuZn SOD antisera. See, Marklund SL, Holme E, Hellner L *Clin Chim Acta* 126;41-51 (1982).

(iii) Procedure

Fructosamine oxidase activity may be measured using the redox-active colour reagent, ferricytochrome c, which is readily reduced by superoxide to form ferrocytochrome c with a characteristic increase in absorbance at 550nM (ϵ_{550} = 22.1 $mM^{-1}.cm^{-1}$). The reagent is 50mM TES buffer pH 7.4 containing 10µM cytochrome c (Sigma), and 50µM fructosamine as glycated bovine serum albumin. The parameters for performance of the assay in a Cobas Bio (Roche) automated analyser are as shown in Table 2.

TABLE 2

	PARAMETER LISTING	
1	UNITS	U/L
2	CALCULATION FACTOR	473.9
3	STANDARD 1 CONCENTRATION	0
4	STANDARD 2 CONCENTRATION	0
5	STANDARD 3 CONCENTRATION	0
6	LIMIT	0
7	TEMPERATURE [DEG.C]	30.0
8	TYPE OF ANALYSIS	6
9	WAVELENGTH [NM]	550
10	SAMPLE VOLUME [UL]	5
11	DILUENT VOLUME [UL]	45
12	REAGENT VOLUME [UL]	200
13	INCUBATION TIME [SEC]	300
14	START REAGENT VOLUME [UL]	25
15	TIME OF FIRST READING [SEC]	0.5
16	TIME INTERVAL [SEC]	300
17	NUMBER OF READINGS	2
18	BLANKING MODE	1
19	PRINTOUT MODE	1

One unit of enzyme was defined as the amount which reduced 1 µmol/minute of cytochrome c in solution under the above assay conditions. The calculation factor is determined from the molar absorptivity for ferrocytochrome c (ϵ_{550nm}) according to the formula:

$$U/L\ (\mu mol.min^{-1}.L^{-1}) = TV \times 10^3 / \epsilon_{550} \times SV$$

where TV = total reaction volume

SV = sample volume

(iv) Materials

Glycated bovine serum albumin substrate was prepared as follows:

(a) Bovine serum albumin (BSA) (Sigma) was reduced with sodium borohydride to eliminate protein hydroperoxides. BSA (60g/L) was dissolved in 0.145M NaCl, pH was adjusted to 9.0 with molar NaOH, sodium borohydride (200mmol/L) was added, and the solution was stirred gently at room temperature for 24 hours. Excess sodium borohydride was discharged with glacial acetic acid and the solution was dialysed exhaustively against 0.145M NaCl at 4 °C.

(b) Borohydride-reduced BSA was glycated by mixing protein solution with an equal volume 0.4M Na_2PO_4 buffer pH 7.4 containing 50mM glucose and 0.02% sodium azide and incubating at 37 °C for 7 days. Excess glucose was removed by exhaustive dialysis against 0.145M NaCl.

(c) Glycated BSA (gBSA) was acetylated by adding 0.2M iodoacetic acid, adjusting pH to 6.8, and incubating at room temperature for 24 hours. Excess iodoacetate was removed by exhaustive dialysis against 0.145M NaCl.

(d) Remaining copper binding sites on gBSA were saturated by dialysing against 0.145M NaCl containing 100μM copper sulphate. Excess copper was removed by exhaustive dialysis against 0.145M NaCl.

(e) Degree of glycation of gBSA substrate was determined by fructosamine assay (Hoffmann La-Roche).

(v) Substrate specificity

The specificity of the assay for reactive oxygen species was tested by measuring the degree of inhibition of ferricytochrome c reduction after adding the following oxygen free radical scavengers to the reaction mixture: (a) Superoxide dismutase to selectively remove superoxide; (b) catalase to selectively remove hydrogen peroxide; & (iii) mannitol to scavenge hydroxyl radicals. Results are shown in Table 3.

TABLE 3

Free radical scavenger	Enzyme activity* (U/L)	Significance (P)
Control	15.34 ± 0.16	-
superoxide dismutase (20kU/L)	9.99 ± 0.03	<0.0001
catalase (1000kU/L)	12.23 ± 0.03	<0.0001
superoxide dismutase + catalase	6.78 ± 0.12	<0.0001
mannitol (50mmol/L)	14.96 ± 0.19	0.0421

- determined with free radical scavenger added to the reagent. Results imply that the assay reaction is measuring both superoxide and hydroxyl radicals formed from the reaction of superoxide with hydrogen peroxide.

(v) Specificity

Cytochrome c is a non-specific reductant and other reducing substances in sera or anticoagulants added to the blood sample at specimen collection may interfer in the assay as shown in Table 4.

TABLE 4

Additive*	Activity compared with control (%)
Control	100
Heparin (1000U/L)	24.4
EDTA (100µM)	26.3

- Human *fructosamine oxidase* analysed with and without (control) additive in the reagent

(vi) Comparison with fructosamine concentrations

Fructosamine oxidase activity was measured in non-diabetic sera and results were compared with serum fructosamine concentrations Figure 2.

(vii) Identifying *fructosamine oxidase* inhibitors

An important application of the current activity assay is as a means to identify potential *fructosamine oxidase* antagonists and inhibitors . *Fructosamine oxidase* inhibitors may be hydrazine compounds which bind and block the quinone co-factor, copper chelators which bind and block the copper co-factor, or substrate analogues which mimic the normal substrate of the enzyme. Micromolar amounts of candidate

substance are added to the reaction mixture, and the decrease in *fructosamine oxidase* activity of a human plasma sample is measured. The inhibitory potential of carbidopa (hydrazine compound), potassium cyanide (copper chelator), and captopril (substrate analogue) are demonstrated in FIGURE 3.

The effectiveness of an enzyme inhibitor is usually expressed by a velocity constant (K) which determines the fraction of the enzyme inhibited in a given period of time by a certain concentration of inhibitor. The specificity of the inhibitor for the active centre of the enzyme is indicated by the concentration of inhibitor causing 50% inactivation of the enzyme (IC_{50}). Results of this *in vitro* assay would suggest that, at 1μM inhibitor concentration, the most effective enzyme inhibitor is carbidopa (K = 15% per minute) followed by captopril (K = 2.6% per minute) followed potassium cyanide (K = 1.2% per minute). Carbidopa also shows the greatest specificity for the active centre of *fructosamine oxidase* (IC_{50} = 0.50μM) compared with captopril (IC_{50} = 0.83μM) and potassium cyanide (IC_{50} = 6.36μM).

WHAT WE CLAIM IS:

1. A method of determining fructosamine oxidase activity in blood plasma of mammalian patients or a mammalian patient to determine patients or a patient at risk to vascular damage, which method comprises determining the levels of fructosamine oxidase and/or the superoxide reaction product of fructosamine oxidase and/or any other oxygen free radical product of fructosamine oxidase in the population of patients and making the determination dependant upon such levels.

2. A method of claim 1 wherein the patients are humans suffering from or predisposed to diabetes.

3. A method of claim 1 or 2 wherein said fructosamine oxidase activity is measured in blood taken from each patient.

4. A method of claim 3 wherein the measurement conducted *in vitro* is of the superoxide reaction product or any other oxygen free radical product of fructosamine oxidase.

5. A method of any one of the preceding claims wherein at risk patients are or an at risk patient is then treated inter alia to inhibit and/or to antagonise fructosamine oxidase.

6. A method of identifying those individuals who will benefit by treatment with fructosamine oxidase inhibitors and/or antagonists, which method comprises testing an individual or a group of individuals for fructosamine oxidase in their blood directly or by reference to the superoxide reaction product of fructosamine oxidase or any other oxygen free radical product of fructosamine oxidase.

7. A method of claim 6 wherein at risk patients are then treated inter alia to inhibit and/or to antagonise the fructosamine oxidase.

8. A method of monitoring fructosamine oxidase inhibition and/or antagonism of a patient which comprises or includes testing directly or indirectly the fructosamine oxidase level of such patient.

9. A method of claim 8 wherein such testing is by reference to the superoxide reaction product of fructosamine oxidase any other oxygen free radical product of fructosamine oxidase in the blood of the patient.

10. A method of any one of the preceding claims which involves a determination of a

particular level attributed to fructosamine oxidase and/or the reaction products referred to in comparison to such level or levels of a patient or patients (as the case may be) who is or are not at risk to such vascular damage, or will not benefit by treatment with fructosamine oxidase inhibitors and/or antagonists or have no need for fructosamine oxidase inhibition and/or antagonism.

11. A method of testing and/or identifying fructosamine oxidase inhibitors or a fructosamine oxidase inhibitor which comprises measuring the effect a candidate substance has or candidate substances have on one or more of the quinone co-factor, the copper co-factor, or at least one substrate analogue of fructosamine oxidase.

12. A method of identifying a candidate substance for trial for the amelioration of diabetes induced vascular damage in a mammal which comprises testing such a substance for fructosamine oxidase inhibition and/or antagonism and choosing to trial the substance where

(ii) it has a specifity for such an enzyme, or its co-factors,

and

(iii) it has an effectiveness for such inhibition and/or antagonism at dosage levels not known to be toxic or contraindicated in such a mammal.

13. The measurement *in vitro* of the superoxide reaction product (and/or any other oxygen free radical product) of fructosamine oxidase in the blood of a mammal by exploiting its reductant properties or its oxidant properties or by enzymatic means.

14. The measurement of claim 13 wherein the procedure involves the disabling of the superoxide scavenging mechanism (such as superoxide dismutase) (SOD) and then exposure to a suitable fructosamine oxidase substrate.

15. The measurement of claim 14 wherein it is made at a pH of 7 to 8.

16. The measurement of claim 15 wherein the pH is at greater than 7.5.

17. The measurement of claim 16 wherein the disabling is by pre-treatment of the plasma sample with anti-human CuZn SOD antisera.

18. The measurement of claim 16 wherein the fructosamine oxidase substrate is glycated bovine serum albumin modified to eliminate copper chelating activity which might diasable the fructosamine oxidase.

19. The measurement of claim 16 which involves measurement of an absorbance

change, chemiluminescent change, or some other characterising change in an indicator of the modified sample.

20. A method of determining the fructosamine oxidase levels in a mammal which involves the measurement as claimed in claim 19.

21. A method of determining the blood plasma fructosamine oxidase levels in a diabetic individual or a suspected diabetic individual which involves the measurement procedure of claim 19.

22. A method of assaying blood serum or blood plasma *in vitro* directly and/or indirectly for fructosamine oxidase which involves the measurement procedure of claim 19.

23. A method of claim 11 or 12 which involves a method of any one of claims 13 to 22.

24. A blood serum or blood plasma sample of a patient in which the superoxide scavenging mechanisms therein have been disabled and the pH is in the range from 7 to 8.

25. A sample of claim 24 which has been modified by exposure to a suitable fructosamine oxidase substrate.

26. A sample of claim 25 wherein said fructosamine oxidase substrate is glycated bovine serum albumin modified to eliminate copper chelating activity which might disable fructosamine oxidase.

27. The use of a sample as claimed in any one of claims 24 to 26.

ABSTRACT

Methods whereby, by reference to fructosamine oxidase activity in blood plasma of a patient or patients, the risk of diabetes associated vascular complications can be assessed, candidate fructosamine oxidase inhibitors and/or antagonists can be identified or tested and the inhibition and/or antagonism of the fructosamine oxidase inhibition and/or antagonsim of a patient can be assessed.

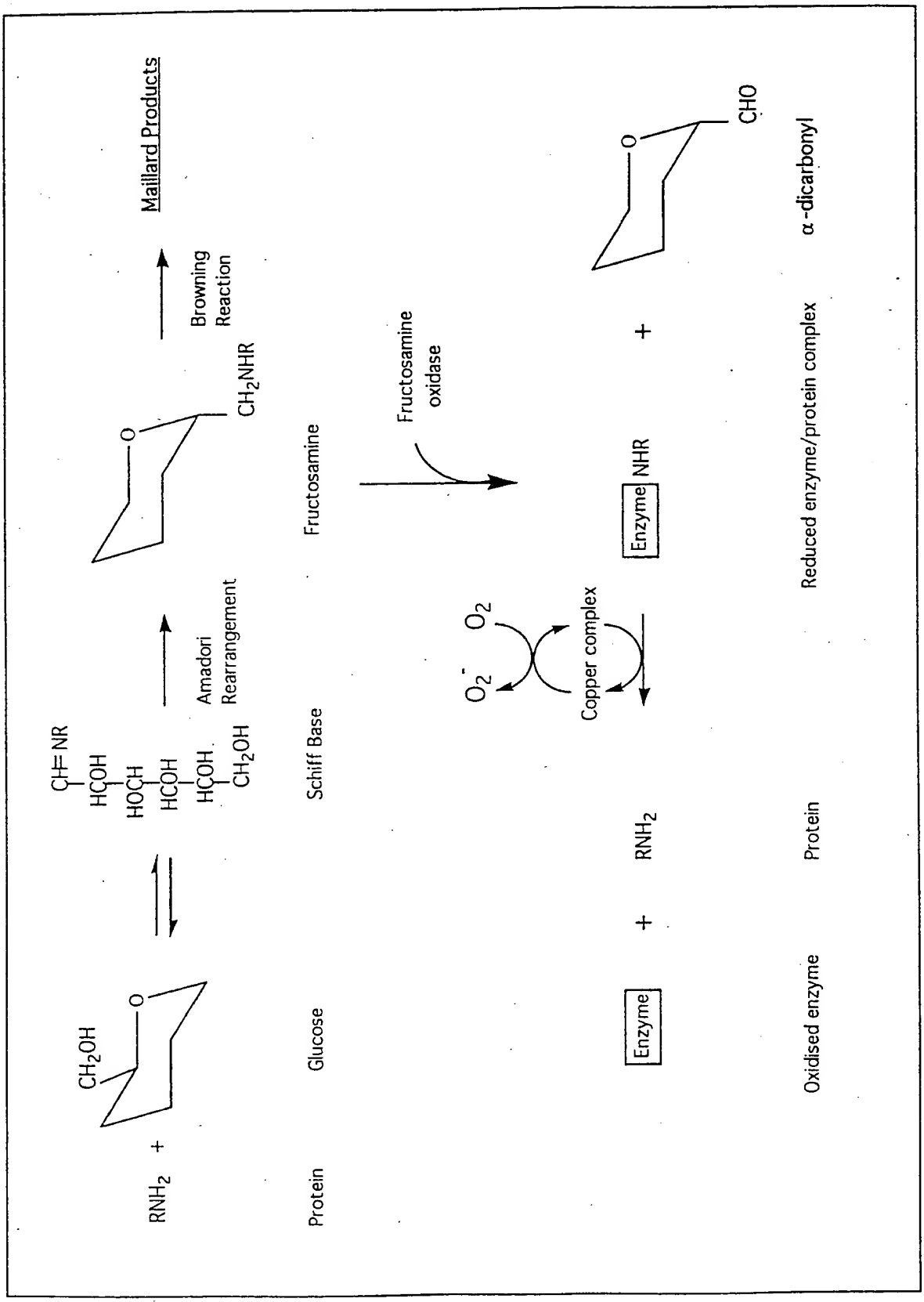
Maillard Products
Browning Reaction
Amadori Rearrangement
Fructosamine oxidase
Copper complex
Protein
Glucose
Schiff Base
Fructosamine
Enzyme
Oxidised enzyme
Protein
Reduced enzyme/protein complex
α -dicarbonyl

FIGURE 1.

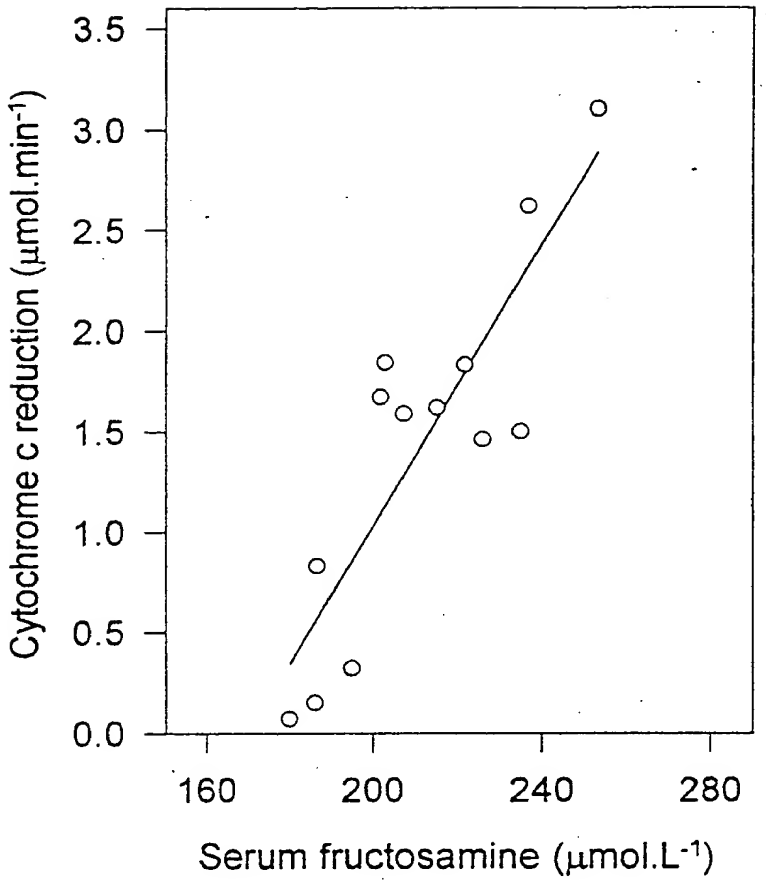
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cytochrome c reduction (µmol.min-1)
160
200
240
280
Serum fructosamine (µmol.L-1)

FIGURE 2.

Inhibitors of *fructosamine oxidase* in plasma

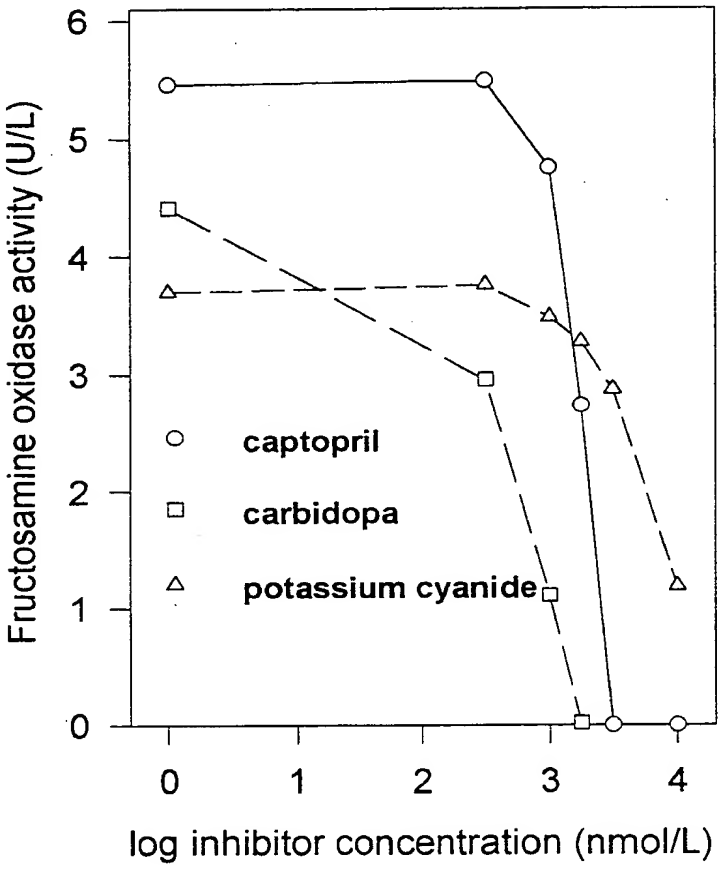

FIGURE 3.